Elevated Circulation Levels of an Antiangiogenic SERPIN in Patients with Diabetic Microvascular Complications Impair Wound Healing through Suppression of Wnt Signaling

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Wound healing, angiogenesis, and hair follicle maintenance are often impaired in the skin of diabetic patients, but the pathogenesis has not been well understood. Here, we report that circulation levels of kallistatin, a member of the serine proteinase inhibitor (SERPIN) superfamily with antiangiogenic activities, were elevated in type 2 diabetic patients with diabetic vascular complications. To test the hypothesis that elevated kallistatin levels could contribute to a wound-healing deficiency via the inhibition of Wnt/β-catenin signaling, we generated kallistatin-transgenic (KS-TG) mice. KS-TG mice had reduced cutaneous hair-follicle density, microvascular density, and panniculus adiposus layer thickness, as well as altered skin microvascular hemodynamics and delayed cutaneous wound healing. Using Wnt reporter mice, our results showed that Wnt/β-catenin signaling is suppressed in the dermal endothelium and hair follicles in KS-TG mice. Lithium, a known activator of β-catenin via inhibition of glycogen synthase kinase-3β, reversed the inhibition of Wnt/β-catenin signaling by kallistatin and rescued the wound-healing deficiency in KS-TG mice. These observations suggest that elevated circulating antiangiogenic serpins in diabetic patients may contribute to impaired wound healing through inhibition of Wnt/β-catenin signaling. Activation of Wnt/β-catenin signaling, at a level downstream of Wnt receptors, may ameliorate the wound-healing deficiency in diabetic patients.

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INTRODUCTION

Globally, every 30 seconds, a limb is amputated owing to pathologic complications associated with diabetes mellitus (Tseng, 2006; Rajamani *et al.*, 2009; Margolis *et al.*, 2011). There is a strong clinical need to identify biomarkers or therapeutic targets in the circulation and skin that modulate skin maintenance and repair in diabetes.

In humans, 20 extracellular serine proteinase inhibitors (SERPINs) comprise ~10% of proteins by mass in the human circulation (Irving *et al.*, 2000; Goettig *et al.*, 2010). Serpins α1-antitrypsin (*SERPINA1*), pigment epithelium–derived factor (*SERPINF1*), and kallistatin (*SERPINA4*) have displayed anti-angiogenic activities (Dawson *et al.*, 1999; McMahon *et al.*, 2001; Miao *et al.*, 2002). Recently, we have shown that kallistatin binds with low-density lipoprotein receptor–related protein 6 (LRP6), an essential coreceptor of the canonical Wnt pathway, and suppresses the activation of Wnt signaling by Wnt ligands (Liu *et al.*, 2013). Here, we explore the concept that kallistatin regulates skin hair follicle development and wound healing through interactions with the canonical Wnt signaling pathway.

Canonical Wnt signaling in adult tissues upregulates the expression of direct T-cell factor (TCF)/lymphoid enhancer factor-1 target genes that modulate hair follicle growth (DasGupta and Fuchs, 1999), cell proliferation (He *et al.*, 1998), and angiogenesis (Zhang *et al.*, 2001). Wnt ligands,

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Abbreviations: HDMVEC, human dermal microvascular endothelial cell; KS-TG, kallistatin transgenic; LCM, L-cell-conditioned medium; LiCl, lithium chloride; LRP6, lipoprotein receptor–related protein 6; PA, panniculus adiposus; PC, panniculus carnosus; P0, postnatal day 0; SERPIN, serine proteinase inhibitor; TCF, T-cell factor; WCM, Wnt3a-conditioned medium; WT, wild type; X-gal, 5-Bromo-4-chloro-3-indolyl b-D-galactopyranoside

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such as Wnt3a, bind to a coreceptor complex consisting of frizzled receptors and LRP6, causing phosphorylation of LRP6 and recruitment of a degradation complex consisting of casein kinase 1, glycogen synthase kinase-3β, and adenopolyposis coli protein. In the absence of Wnt ligand, this kinase complex phosphorylates β-catenin, leading to degradation of β-catenin in the cytoplasm (MacDonald *et al.*, 2009). Phosphorylation and degradation of β-catenin is prevented when Wnt ligands activate the pathway, and the stabilized β-catenin translocates to the nucleus and dimerizes with the TCF–Groucho complex, activating the transcription of corresponding direct target genes. Canonical Wnt signaling is crucial for development and has key roles in cancer progression (MacDonald *et al.*, 2009); yet, its role in wound healing has only recently been studied (Fathke *et al.*, 2006; Wu *et al.*, 2011; Whyte *et al.*, 2012, 2013). Although Wnt signaling has been shown to promote angiogenesis (Parmalee and Kitajewski, 2008; Zerlin *et al.*, 2008; Barcelos *et al.*, 2009; Chen *et al.*, 2009; Phng *et al.*, 2009; Dejana, 2010; Zhang and Ma, 2010; Chen *et al.*, 2011) and be essential for the morphogenesis of hair follicles (Ito *et al.*, 2007; Enshell-Seijffers *et al.*, 2010), the role of antiangiogenic serpins in modulating Wnt signaling and wound healing in adult skin has not been investigated. We undertook this study to explore the role of kallistatin in modulation of wound healing and identified a potential pharmacological rescue strategy to attenuate the negative effects of antiangiogenic serpins in wound healing.

RESULTS

Elevation of serum kallistatin levels in type 2 diabetic patients with vascular complications

We analyzed kallistatin levels in the sera of healthy individuals and type 2 diabetic patients with or without clinically evident diabetes-related vascular complications (Supplementary Table S1 online). In diabetic patients with complications, 87.5% of patients had microvascular complications, including 46.9% with peripheral neuropathy (Supplementary Table S1 online). Circulating kallistatin levels differed significantly across the three subject groups (Figure 1). Kallistatin levels were significantly higher in the type 2 diabetic patients with vascular complications compared with nondiabetic control subjects and diabetic patients without complications (Figure 1). Kallistatin levels in diabetic patients showed correlations with various clinical parameters related to vascular health, including HbA1c, albumin-to-creatinine ratio, large-artery elasticity, and small-artery elasticity (Supplementary Table S2 online).

Reduced hair-follicle density and skin microvascular density in kallistatin-transgenic (KS-TG) mice

To understand the impacts of elevated kallistatin levels, we generated KS-TG mice overexpressing and secreting human kallistatin into the circulation and tissues (Supplementary Figure S1a and b online). Circulating levels of endogenous mouse kallistatin (SERPINA3C) were approximately 1 μg ml^{-1} in wild-type (WT) mice (Supplementary Figure S1c online), whereas KS-TG mice had circulating levels of kallistatin at 5 μg ml^{-1} (Supplementary Figure S1d online). The increase of

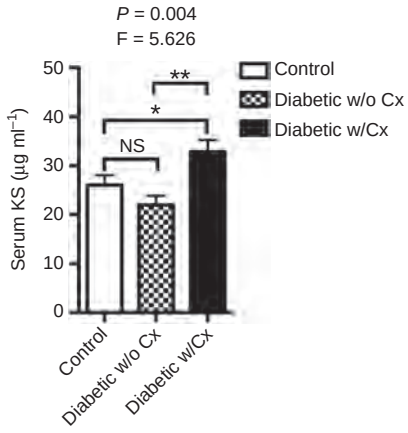

Figure 1. Elevation of serum kallistatin (KS) levels in type 2 diabetic patients with vascular complications of diabetes. Nondiabetic subjects ($N=45$), diabetic patients without vascular complications (DM w/o Cx, $N=36$), and diabetic patients with vascular complications (DM w/Cx, $N=44$). Mean ± SEM, analysis of variance: $P=0.004$, $F=5.626$. *Post hoc* analysis group-versus-group comparison indicated with bars. *$P<0.05$, **$P<0.01$. NS, not significant.

serum levels of human kallistatin in KS-TG mice were comparable to the fold increase of kallistatin in diabetic patients with vascular complications. The elevated kallistatin levels were detected in the skin of KS-TG mice at sufficient amounts for studying a potential skin phenotype (Supplementary Figure S1d online).

In vitro tissue kallikrein activity assays showed that KS-TG mice had no detectable change in tissue kallikrein activity in wounded skin or serum, compared with the WT mice (Figure 3j and Supplementary Figure S1e online, respectively). Comparing the amino-acid sequence of human kallistatin (*SERPINA4*) with endogenous mouse kallistatin (*SERPINA3C*) revealed that the sequence of the reactive center loop of human kallistatin that interacts with and is cleaved by tissue kallikrein is not identical to the sequence in mouse kallistatin (Supplementary Figure S1f online), suggesting that human kallistatin probably does not significantly impact the activity of mouse tissue kallikrein.

Newborn KS-TG mice at postnatal day 0 (P0) had reduced hair-follicle density compared with WT littermates (Figure 2a and b; quantification in Figure 2i). Skin with telogen-phase follicles in 3-month-old KS-TG mice showed significantly decreased dorsal skin thickness (Figure 2c and d; quantification in Figure 2k) and reduced hair-follicle density (Figure 2j). Furthermore, as seen after synchronized induction of anagen by depilation, induction of anagen-phase hair follicles was attenuated in KS-TG mice relative to WT littermates (Supplementary Figure S4 online). The decrease in 3-month-old KS-TG mouse skin thickness was largely because of a decrease in the thickness and cell population in the panniculus adiposus (PA) layer, with nuclei numbers in the dermis and panniculus carnosus layers being similar (Figure 2e, f and l). The microvascular density in the skin of KS-TG mice was significantly reduced (Figure 2g and h; quantification in Figure 2m).

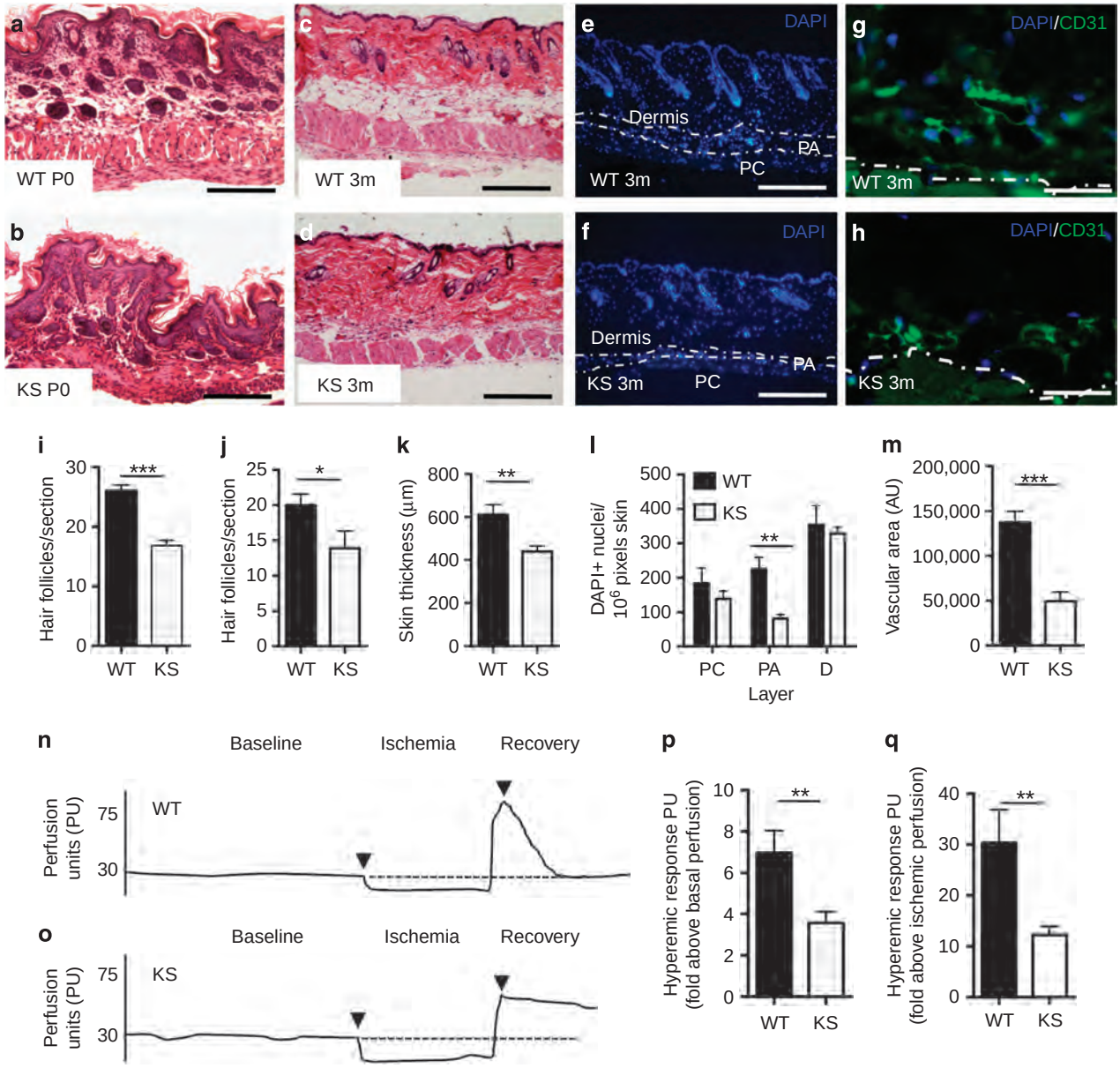

Figure 2. Kallistatin (KS) affects skin structure and function. (**a**–**d**) Hematoxylin and eosin, dorsal skin; (**a**, **b**) newborn postnatal day 0 (P0) wild-type (WT) and kallistatin-transgenic (KS-TG) littermates, scale bar = 100 μm; (**c**, **d**) 3-month-old littermates, scale bar = 500 μm. (**e**, **f**) 4,6-Diamidino-2-phenylindole (DAPI) staining; dotted lines indicate boundaries of the skin dermis, panniculus adiposus (PA), and panniculus carnosus (PC) layers; scale bar = 500 μm. (**g**, **h**) FITC-anti-CD31 antibody, scale bar = 50 μm. (**i**, **j**) Hair-follicle density at P0 (**i**) and 3 months (**j**); (**k**) skin thickness; (**l**) nuclei between dotted lines in PC, PA, dermis (D); (**m**) microvascular density. (**n**, **o**) Laser Doppler flowmetry in hindlimb skin, (**n**) WT and (**o**) KS-TG mice. (**p**, **q**) Hyperemic responses. $N = 5$ or > 5 in all analyses with multiple sections/tissues per analysis. Mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. AU, arbitrary fluorescence units.

Kallistatin overexpression impairs skin hyperemic response to ischemia

Pressure to the skin causes local ischemia. Upon release of pressure, blood flow increases immediately, peaks and rapidly stabilizes due to vascular reactivity (Tur *et al.*, 1991). We measured skin blood flow after standardized local ischemia in adult WT and KS-TG mice (Figure 2n and o). WT skin blood flow dynamics appeared as expected, with a sharp rise and rapid return to baseline (Figure 2n). KS-TG mice, however, had a blunted response above both baseline levels and ischemia levels (Figure 2o; quantified in Figure 2p and q) and had a delay in returning to baseline (Figure 2o).

KS-TG mice have delayed skin wound repair

Skin wound-healing assay demonstrated that wound closure in KS-TG mice lagged behind WT littermates (Figure 3a). Vascular density in the wound area was reduced in KS-TG mice compared to WT mice at day 7 of wound healing, a peak phase of endothelial cell proliferation during wound healing (Nissen *et al.*, 1998; Figure 3d, e, h, and i). There was no

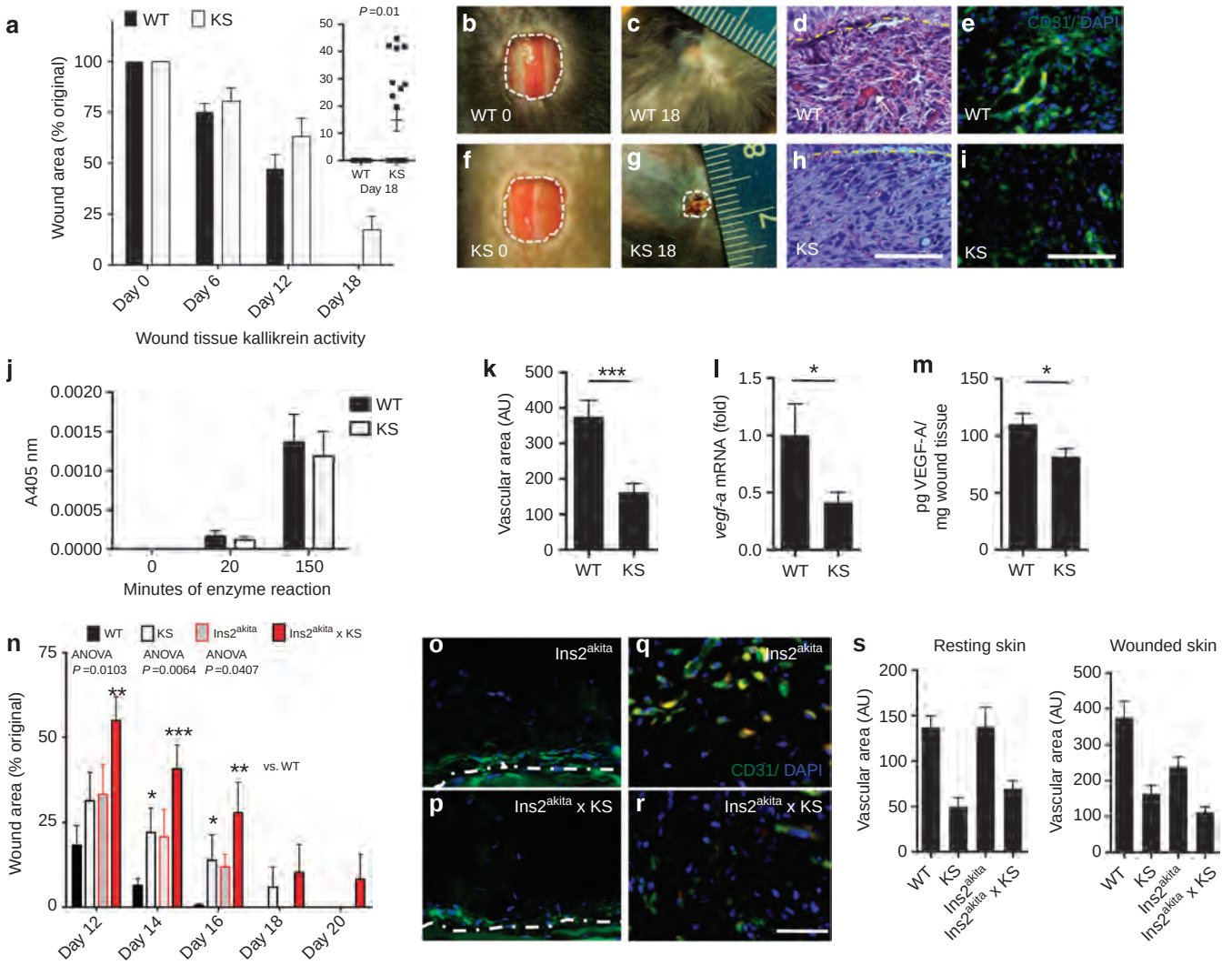

Figure 3. Kallistatin (KS) delays wound closure and inhibits wound angiogenesis. (**a**) Wound-healing rate (3-month-old male littermates). (**b**, **c**, **f**, **g**) Images of representative wounds. (**d**, **h**) Hematoxylin and eosin, wound bed at day 7, scale bar = 50 μm; (**e**, **i**) CD31, wound beds; (**j**) normalized tissue kallikrein activity in wounds; (**k**) wound vascular area; (**l**) *vegf-a* mRNA levels in wounds; (**m**) VEGF-A in wound homogenates; (**n**) wound areas in 3-month-old male mice; (**o**, **p**) $CD31^+$ cells in resting skin in $Ins2^{akita}$ and $Ins2^{akita}$ × kallistatin-transgenic (KS-TG) mice; (**q**, **r**) $CD31^+$ endothelial cells, wounded skin, $Ins2^{akita}$ and $Ins2^{akita}$ × KS-TG mice. Scale bar (**o**–**r**) = 50 μm. (**s**) $CD31^+$ area. Mean ± SEM, $N = 5$ or >5 in all analyses with multiple sections/tissues per analysis, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. AU, arbitrary fluorescence units.

difference in tissue kallikrein activity in wounds at day 7 (Figure 3j). Vascular density in wound beds at day 7 was significantly decreased in KS-TG mice (Figure 3k). The expression of *vegf-a* was significantly lower at both the mRNA and protein levels in KS-TG mice versus WT mice during day 7 of wound healing (Figure 3l and m).

Kallistatin overexpression exacerbates wound-healing delay in diabetic mice

$Ins2^{akita}$ mice represent a model of diabetes caused by an insulin 2 gene mutation (Wang *et al.*, 1999). Although KS-TG mice and $Ins2^{akita}$ mice (3 months old) alone showed mild delays in skin wound healing, $Ins2^{akita}$ × KS-TG mice had the slowest wound healing rate of all groups (Figure 3n). Although having thinner skin than WT, $Ins2^{akita}$ mice had better angiogenic responses in wounds compared with $Ins2^{akita}$ × KS-TG mice, at the age of 3 months (Figure 3o and p are resting skin; Figure 3q and r are wounds; quantification in Figure 3s).

KS-TG mice have reduced activation of Wnt/TCF/β-catenin signaling in skin and wounds

We examined whether kallistatin overexpression affects Wnt/TCF/β-catenin signaling in the skin by crossing KS-TG mice with Wnt/TCF/β-catenin-reporter BAT-gal mice, which express the β-galactosidase reporter gene under the control of a promoter containing TCF/β-catenin-binding sites. 5-Bromo-4-chloro-3-indolyl b-D-galactopyranoside staining indicated that Wnt signaling was activated in the periphery of the wounded skin (Figure 4a and b) and in cells that had an endothelial-like

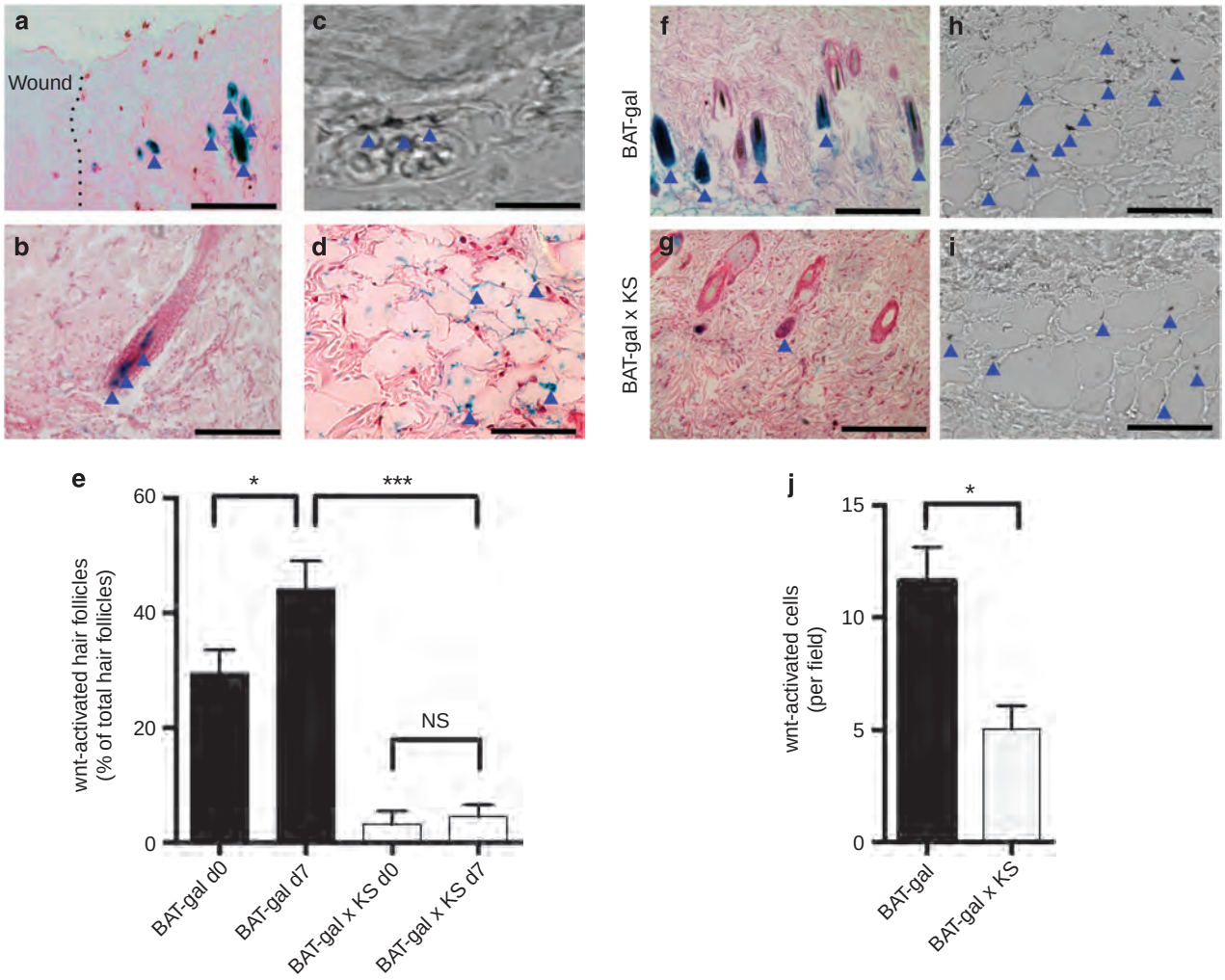

Figure 4. Kallistatin (KS) is associated with a reduction in Wnt signaling in hair follicles and during wound healing. (**a**) 5-Bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal)-stained hair follicles surrounding the wound area, Wnt-reporter BAT-gal mice. (**b**) Wnt activation in various positions in the hair follicle adjacent to the wound. (**c**) differential interference contrast image, X-gal$^+$ endothelial cell in skin. (**d**) X-gal$^+$ endothelial cells, wound bed. (**e**) Quantification, X-gal$^+$ hair follicles. (**f**, **g**) X-gal$^+$ hair follicles surrounding wounds. (**h**, **i**) X-gal$^+$ cells, day 7 wound beds. (**j**) Quantification of X-gal$^+$ cells. In all panels, blue arrows indicate X-gal staining. Scale bars = (**a**) 200 μm, (**b**) 100 μm, (**c**) 25 μm, (**d**) 50 μm, (**f**, **g**) 200 μm, (**h**, **i**) 50 μm. $N = 5$ or >5 in all analyses with multiple sections/tissues per analysis. Mean ± SEM, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. ANOVA, analysis of variance; AU, arbitrary fluorescence units; NS, not significant; WT, wild type.

morphology and were co-stained with CD31 (Figure 4c and d) in the wound beds and hair follicles of BAT-gal mice. In the wounded Wnt reporter mice, the number of hair follicles in the immediate periphery of the wound with Wnt reporter activity was significantly higher than in resting skin (Figure 4e). The resting skin of BAT-gal mice had more than 25% of the total population of hair follicles with Wnt activation, whereas resting skin of BAT-gal × KS-TG mice had <10% of hair follicles with Wnt signaling activation (Figure 4e). During the proliferative phase of wound healing, BAT-gal mice had over 45% of Wnt-activated hair follicles adjacent to the wound area, whereas BAT-gal × KS-TG mice had <10% activation (Figure 4e). In wound beds in the proliferative stage, BAT-gal mice had higher densities of cells with active Wnt signaling (Figure 4h), and these cells were associated with CD31 in wound beds, compared with BAT-gal × KS-TG mice (Figure 4i; quantification in Figure 4j).

Kallistatin inhibits Wnt/β-catenin signaling in primary human dermal microvascular endothelial cells

To dissect the effect of kallistatin on endothelial Wnt signaling, we treated primary human dermal microvascular endothelial cells (HDMVECs) with 30% Wnt3a-conditioned medium (WCM) or with L-cell-conditioned medium (LCM) as control. In an *in vitro* angiogenesis assay, kallistatin reduced the WCM-induced tube and branch formation from HDMVECs after a 12 hour treatment (Figure 5a–c). WCM stimulated HDMVEC proliferation over 72 hours, compared with LCM control (Figure 5d). Purified kallistatin inhibited WCM-induced proliferation of the dermal microvascular endothelial

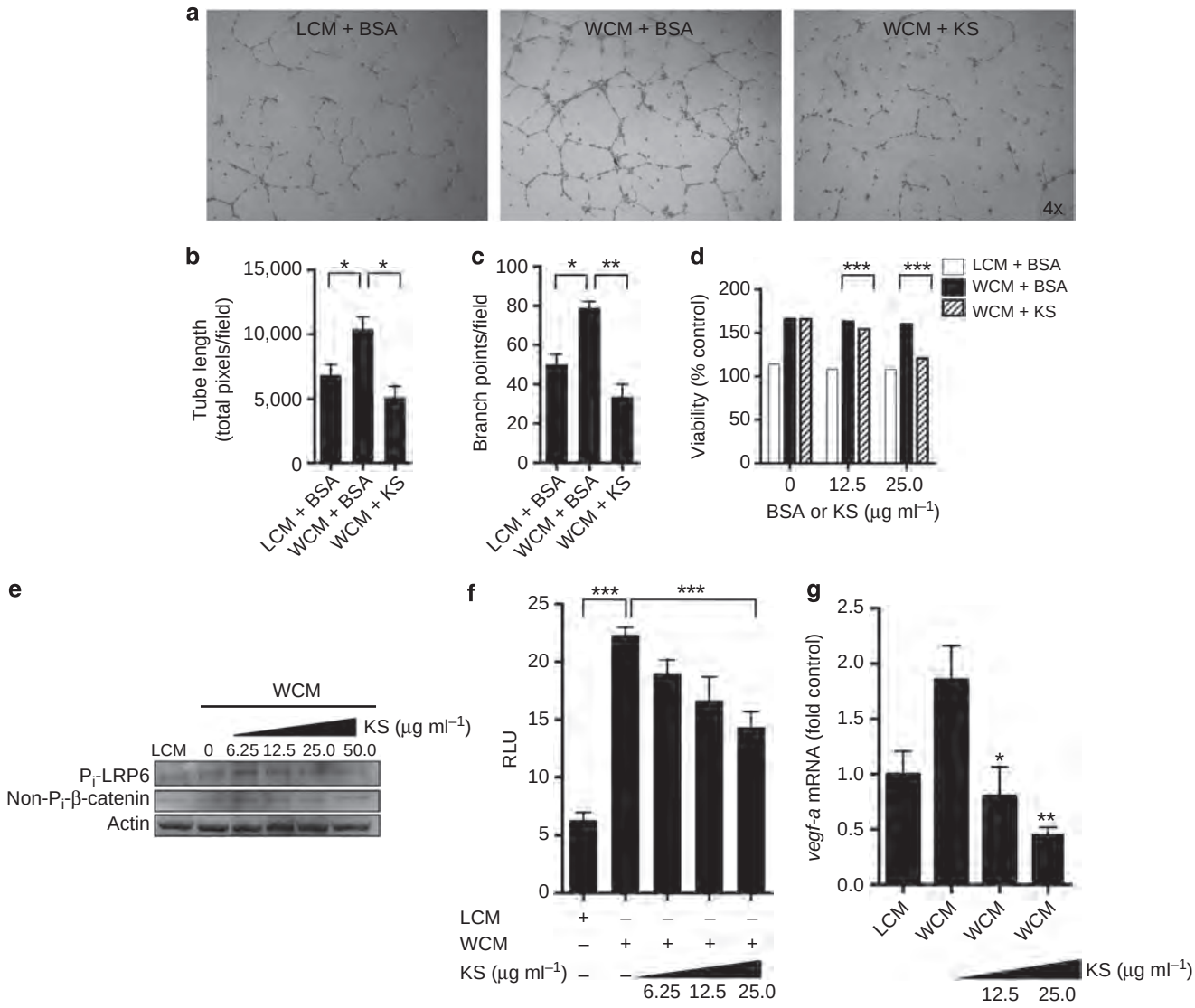

Figure 5. Kallistatin (KS) reduces Wnt3a-induced dermal endothelial cell angiogenesis and Wnt3a-induced T-cell factor (TCF)/β-catenin-dependent transcription. *In vitro* angiogenesis assay, primary human dermal microvascular endothelial cells (HDMVECs). (**a**) 30% L-cell-conditioned medium (LCM) + 25 μg ml^{-1} BSA, 30% Wnt3a-conditioned medium (WCM) + 25 μg ml^{-1} BSA, 30% WCM + 25 μg ml^{-1} KS. (**b**) Total tube length quantification; (**c**) branch points, (**d**) HDMVECs treated simultaneously with 30% WCM and purified KS or BSA, 48 hours. Cell viability via the MTT assay, (**e**) western blot analysis, phosphorylated lipoprotein receptor-related protein 6 (LRP6; P_i-LRP6); HDMVECs. (**f**) HDMVECs, infected with lentivirus-expressing luciferase driven by TCF/β-catenin (*Renilla* luciferase for normalization). HDMVECs were treated with 30% LCM or 30% WCM and different concentrations of KS for 16 hours. (**g**) *vegf-a* mRNA levels in HDMVECs treated as indicated for 16 hours. Mean ± SEM, *$P<0.05$, **$P<0.01$, ***$P<0.001$.

cells, compared with BSA control (Figure 5d). Kallistatin reduced Wnt3a-induced phosphorylation of LRP6, an essential coreceptor of canonical Wnt signaling, and levels of non-phosphorylated β-catenin in HDMVECs, suggesting an inhibitory effect on Wnt signaling in endothelial cells (Figure 5e). To assess kallistatin's effect on Wnt3a/TCF/β-catenin-dependent transcription in HDMVECs, we delivered vectors via lentivirus for TCF/β-catenin-driven luciferase and constitutively expressed *Renilla* luciferase. Luciferase assay revealed that HDMVECs harbor the endogenous machinery for canonical Wnt signaling and respond to Wnt3a ligand in WCM versus LCM (Figure 5f). Furthermore, kallistatin dose dependently reduced the transcriptional activity of β-catenin in HDMVECs (Figure 5f). Expression of a direct angiogenic Wnt/TCF/β-catenin target gene, *vegf-a*, was shown to be upregulated in HDMVECs by WCM and downregulated by kallistatin (Figure 5g). Taken together, these data support that kallistatin impairs dermal angiogenesis, at least in part, by inhibition of canonical Wnt/TCF/β-catenin signaling in skin endothelial cells.

Lithium attenuates the effects of kallistatin on skin angiogenesis and wound healing

To confirm that the effect of kallistatin on wound healing is through inhibition of Wnt signaling by blocking LRP6, we activated TCF/β-catenin intracellularly via pharmacological

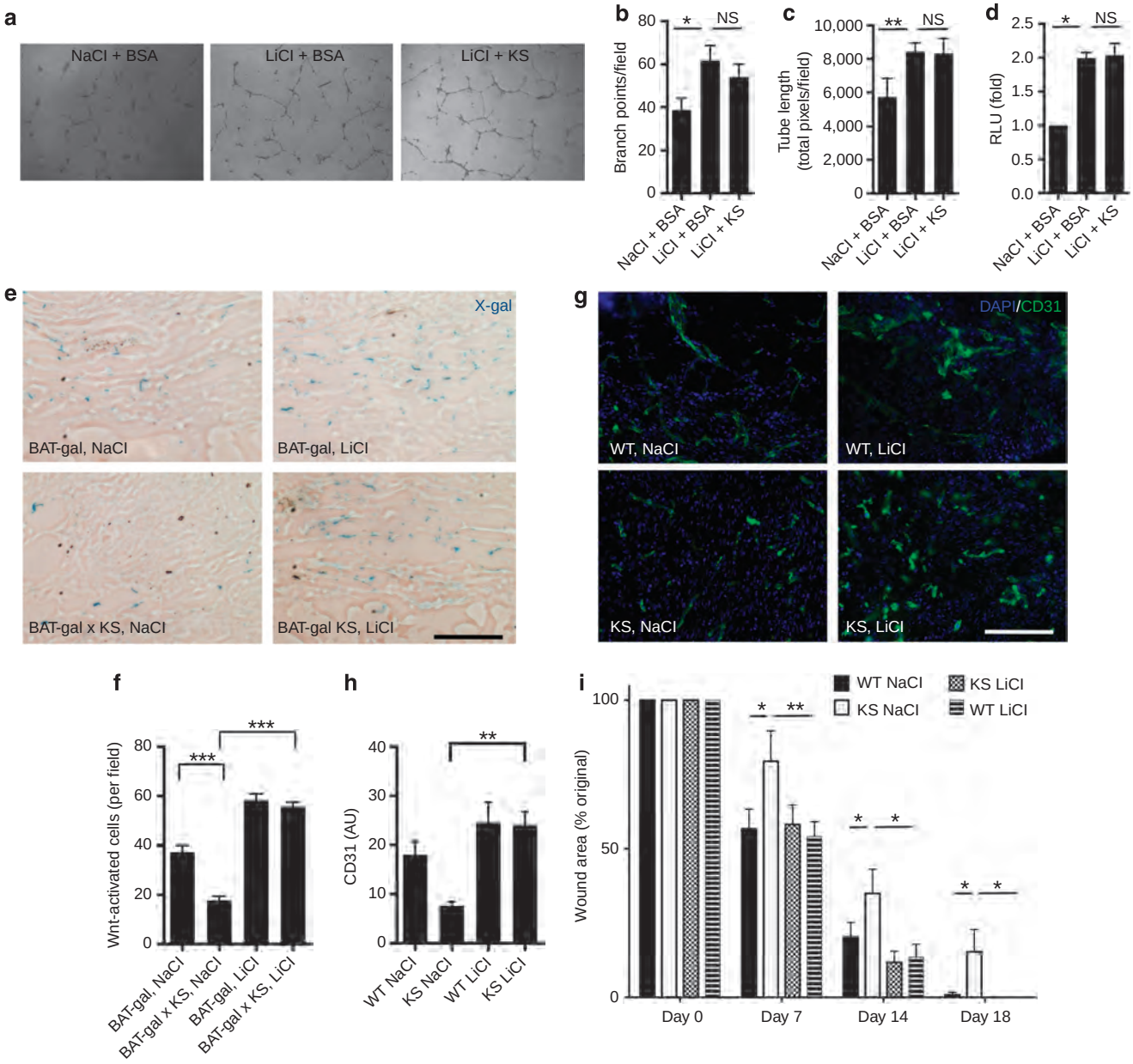

Figure 6. Lithium attenuates the antiangiogenic and anti-Wnt effects of kallistatin (KS). (**a**) Tube formation assay with human dermal microvascular endothelial cells (HDMVECs). (**b**) Total branch points, $n=3$. (**c**) Total tube length, $n=3$. (**d**) TCF/β-catenin transcriptional activity in HDMVECs. NS, not significant. (**e**) X-gal staining showing Wnt activation in day 7 wounds of Wnt reporter mice in response to NaCl or LiCl topical treatments. (**f**) Quantification of Wnt-reporter X-gal activity. (**g**) $CD31^+$ cells in day 7 wound beds after topical treatments. (**h**) Quantification of $CD31^+$ cells, day 7 wound beds. AU, arbitrary fluorescence units. (**i**) Overall skin wound-healing rate expressed by wound area, $N=7$–10 age-matched male mice, $n=5$ or >5 in all analysis with multiple sections/tissues per analysis. Mean ± SEM, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, analysis of variance and Tukey's *post hoc* significance analysis performed. Scale bar (**e**, **g**) = 50 μm.

inhibition of glycogen synthase kinase-3β and subsequent stabilization of β-catenin using lithium chloride (LiCl). HDMVECs formed more branches and longer tubes in the presence of 5 mM LiCl versus 5 mM NaCl (Figure 6a–c). The addition of 25 μg ml^{-1} purified kallistatin was unable to significantly attenuate HDMVEC tube formation induced by 5 mM LiCl (Figure 6a–c). Consistently, the same concentration of kallistatin, although able to reduce Wnt3a-induced TCF/β-catenin-driven transcription and tube formation (Figure 5a–c and f), was unable to decrease TCF/β-catenin-driven transcription induced by LiCl (Figure 6d).

To test whether or not lithium has the capacity to rescue the wound-healing delay associated with kallistatin overexpression, wounded mice were treated topically with 20 mM LiCl in DMEM, applied directly to the wounds, a dose previously shown to activate Wnt signaling in BAT-gal mice *in vivo* (Fathke *et al.*, 2006), twice daily for the first 7 days of wound healing, followed by once daily for days 8–10 of wound

healing. BAT-gal × KS mice treated with topical LiCl showed a robust increase in the numbers of cells with activated Wnt signaling, which were associated with $CD31^+$ areas in the wound, compared with BAT-gal × KS mice treated with topical NaCl (Figure 6e and f). The LiCl treatment of KS-TG mice increased the endothelial cell density in wound beds significantly compared with KS-TG mice treated with 20 mM NaCl DMEM (Figure 6g and h). As a consequence, topical LiCl treatment significantly rescued wound repair in KS-TG mice (Figure 6i).

DISCUSSION

Our study establishes that increased circulating levels of an abundant, endogenous antiangiogenic serpin in patients with diabetic microvascular complications contribute to impaired skin function and wound repair. Kallistatin is secreted by nearly every cell type *in vivo* (Chao *et al.*, 1996); yet, its roles in modulating the structure and physiology of many organs are not fully understood. Kallistatin was originally identified as a specific-binding protein and inhibitor of tissue kallikrein (Chao *et al.*, 1986). Kallistatin is a heparin-binding protein (Chen *et al.*, 2001) and is expressed in a wide array of tissues and cell types, including endothelium, salivary glands, and immune cells (Chao *et al.*, 1996; Wolf *et al.*, 1999). This pattern of expression and secretion, as well as its characterization as an inhibitor of angiogenesis, strongly suggests that kallistatin is involved in the regulation of vascular function and remodeling in skin.

The causes of systemic elevation of kallistatin in diabetic patients with microvascular complications are not yet known. It may be due to increased secretion and/or decreased reuptake by the liver, as the liver has been shown to be the major recycler of the kallistatin–kallikrein complex from the circulation (Xiong *et al.*, 1992). We demonstrated in cell culture that high glucose treatment upregulates kallistatin expression in HepG2 cells, a cell line derived from human liver, but did not find evidence that endogenous mouse kallistatin is elevated in early diabetes in 3-month-old $Ins2^{akita}$ mice (Supplementary Figure S3 online).

Diabetic patients with retinal and renal complications are at higher risks of neuropathy and cardiovascular disease and are more likely to develop foot ulcers and require lower limb amputations (Monteiro-Soares *et al.*, 2012). Here, we show that transgenic elevation of human kallistatin levels in mice affected the ultimate structure and histology of the skin, with resting skin being thinner in the PA layer, having reduced skin microvascular density and less hair follicles—features of human lower limb skin in patients with diabetes and/or peripheral vascular disease. Although thickening of some parts of the skin may occur in diabetic patients, such as with acanthosis nigricans and with diabetic pseudoscleroderma (Kostler *et al.*, 2005), high levels of kallistatin may contribute to what is also often seen in diabetic skin—thinning of the PA layer that harbors the subcutaneous fat and blood vessels (Petrofsky *et al.*, 2008). As the PA layer loses structural integrity and becomes thinner, there may be hair loss, reduced capillary return, neuropathy, ulceration, and gangrene—signs of tissue damage that precede lower limb amputation (Hoyt, 2004; Petrofsky *et al.*, 2008). Recent studies elucidated the cross talk between adipocyte precursor cells, epithelial stem cells, and hair-follicle cycling (Festa *et al.*, 2011; Schmidt and Horsley, 2012). Through kallistatin's inhibition of Wnt/β-catenin signaling within hair follicles and endothelial cells, KS-TG mice likely possess defective crosstalk between hair follicles and adipose tissue. At one level, the decreased hair-follicle units likely result in less stimulation of adipose tissue within the PA layer of the mice. Furthermore, the decreased microvascular density within the PA layer likely results in less support for adipocyte precursors, thus disabling the crosstalk between hair follicles and adipose tissue in coordinating proper skin structure and function.

Diabetic patients are known to have impaired skin blood flow and hemodynamic changes upon pressure or injury to the skin (Petrofsky *et al.*, 2009). We found that overexpression of kallistatin resulted in an impaired hyperemic response to local ischemia. KS-TG mice do not develop hyperglycemia, but they still have impaired local skin hemodynamics, mimicking the defective hemodynamics present in diabetic skin. Furthermore, KS-TG mice displayed delayed wound healing, as well as attenuated wound *vegf-a* expression and wound neovascularization.

Taken together, our data suggest that kallistatin is an endogenous Wnt/β-catenin inhibitor in postnatal murine skin; Wnt signaling is known to be a significant modulator of inflammation and angiogenesis (Masckauchan and Kitajewski, 2006; George, 2008). Furthermore, the skin/hair-follicle phenotypes of KS-TG mice are similar to what was reported in transgenic mice systemically overexpressing DKK-1, a potent and specific inhibitor of the canonical Wnt pathway (Sick *et al.*, 2006).

Our recent study showed that kallistatin inhibits Wnt signaling by blocking LRP6, an essential coreceptor in the canonical Wnt pathway (Liu *et al.*, 2013). To confirm that the impact of kallistatin on wound healing is indeed through the inhibition of Wnt signaling, we activated Wnt signaling downstream of LRP6. Lithium, a drug approved by the Food and Drug Administration to treat mood disorders and known to increase *vegf-a* expression (Kaga *et al.*, 2006; Guo *et al.*, 2009), is a potent activator of canonical Wnt signaling, which acts by inhibiting glycogen synthase kinase-3β and stabilizing β-catenin. Because lithium activates β-catenin downstream of LRP6, and has been shown to rescue vascular defects and restimulate angiogenesis during development (Griffin *et al.*, 2011; Curtis and Griffin, 2012) and in the cardiovascular (Kaga *et al.*, 2006) and central nervous systems (Guo *et al.*, 2009), we chose LiCl as an agent to bypass the blocking effects of kallistatin on Wnt signaling *in vivo* and *in vitro*. Our results showed that LiCl attenuated the effects of kallistatin on wound angiogenesis and wound healing *in vivo* and dermal endothelial tube formation and branching *in vitro*. This experiment provides further evidence supporting that kallistatin causes a wound healing delay through antagonizing LRP6.

We propose the following model: excessive accumulation of antiangiogenic serpins, such as kallistatin, inhibits Wnt/β-catenin signaling, contributing to impaired skin endothelial function and wound healing defects in diabetic patients. Activation of Wnt signaling downstream of Wnt receptors in

the endothelium and hair follicles, in and around wounded skin, may benefit the treatment of impaired wound healing in diabetic patients with elevated levels of antiangiogenic serpins, reducing the overall risk of amputations.

MATERIALS AND METHODS

Human subjects

The study, which adhered to the Declaration of Helsinki Guidelines, was approved by the University of Oklahoma Health Sciences Center Institutional Review Board, and written informed consent was obtained from each subject. History and examination were performed, and clinicians confirmed diabetes-associated vascular complication status before this study. Diabetes-associated complications were predefined as having at least one of the following complications of diabetes: history of leg, foot, or toe amputation, retinopathy, documented myocardial infarction or angina with electrocardiogram changes and/or positive cardiac imaging study, nephropathy, history of TIA or stroke, angioplasty, or vascular bypass surgery.

ELISA specific for kallistatin

Kallistatin levels in sera were quantified by ELISA (R&D Systems, Minneapolis, MN), as previously described (Jenkins *et al.*, 2010). For mouse kallistatin ELISA, wells were coated with 2.0 μg ml^{-1} anti-mouse SERPINA3C antibody (Sinobiological, Daxing, China) overnight, and recombinant SERPINA3C standard (Sinobiological) was used for the standard curve.

Kallistatin-transgenic, diabetic, and Wnt reporter mice

The Institutional Animal Care and Use Committee approved all of the animal experiments described. The chicken β-actin promoter was used to drive systemic expression of human kallistatin cDNA, and cloned into the pTriE × 1.1 vector (Novagen, Darmstadt, Germany).

Tissue kallikrein activity assays

Enzymatic activity of endogenous tissue kallikrein was assayed using the colorimetric substrate S-2266 (Chromogenix, Orangeburg, NY), which can be specifically cleaved by both mouse and human tissue kallikrein. Upon cleavage, the colorimetric reaction produced a yellow color, which was quantified by absorbance at 405-nm wavelength.

Laser Doppler flowmetry

After anesthesia, the hind legs of the mice were fixed in place using mild-adhesive tape, and the laser Doppler probe was fixed firmly to the skin to measure perfusion units using the Perimed PeriFlux System 5000 (Perimed, Stockholm, Sweden).

Skin wound healing assay

Clippers were used on dorsal surface of anesthetized mice to remove hair but retain hair follicles. Standardized circular wounds were made with biopsy punches, and the Image J software (National Institutes of Health) was used to trace wound areas and quantify the pixels within the wound.

Visualization of the transcriptional activity of β-catenin *in vivo*

Skin and wounds from BAT-gal transgenic mice were stained with 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside according to the manufacturer's instructions (Sigma, St Louis, MO).

Dermal microvascular endothelial cell culture and tube formation assay

Primary human dermal microvascular cells were obtained from ATCC (Manassas, VA). The cells were seeded on BD matrigel extracellular matrix mix at a density of 100,000 cells per well in the presence of WCM or LCM as control or 5 mM LiCl in microvascular growth medium (5 mM NaCl as control) and 25 μg ml^{-1} purified His-tagged kallistatin (or 25 μg ml^{-1} BSA as control), and were incubated at 37 °C. Twelve hours post seeding, the tube lengths and branching were imaged under the microscope and quantified to reflect angiogenesis *in vitro*.

Topical application of LiCl during *in vivo* wound healing

During wound healing, sterile 20 mM NaCl or 20 mM LiCl in serum-free DMEM was applied topically to open wounds of single-housed mice (500 μl gently ejected from sterile pipette tips under biosafety hood) twice daily to directly bathe the wounds from days 0 to 7, and once a day from days 7 to 10. Thereafter, the wounds were allowed to heal spontaneously.

Statistics

One-way analysis of variance for continuous variables was used with a Tukey's honest significant difference *post hoc* test for differences between two groups when analysis of variance P-value was <0.05. For animal studies involving two groups, a two-tailed t-test was performed with $P<0.05$ considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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